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Comparative Analysis of Compost Manure and Inorganic Fertilizer on the Bacterial Population Density of Cocoa Seedling Rhizosphere

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ABSTRACT

Soil quality has been known to have a strong effect on cocoa tree growth and the interactions of plant and microbe in the rhizosphere influence plant health, productivity and soil fertility. In this study, the rhizospheres of cocoa seedlings were analyzed for bacterial density population after soil amendment. Cocoa seedlings were self-grown in nursery located at the Orchard of the Faculty of Agriculture, University of Benin and Amelonado variety Tc1-Tc8 pods were used. The seeds were prepared, pre-germinated and planted in bags containing 5 kg top soil. Organic fertilizers (compost poultry manure and cow dung) and inorganic fertilizer was applied to the soil surrounding the seedlings at one month after planting. The sowing soil and the rhizosphere of the cocoa seedlings at one month to four month were collected and analyzed. Serial dilution method was used for culturing and bacterial isolates were identified by Gram staining and various biochemical methods. The isolates were further characterized by DNA extraction, PCR and sequencing. All isolates belong to the phyla Proteobacteria, save one which belongs to the phylum Firmicutes; all of which are predominately found in the soil. The result revealed that the rhizosphere of seedlings amended with inorganic fertilizer recorded the least bacterial activity ($2.35 \times 10^5 - 3.05 \times 10^5$ cfu/g), while that of poultry manure recorded the highest ($8.20 \times 10^5 - 1.17 \times 10^6$). The application of poultry manure showed a significant difference in the bacterial population.

1. Introduction

Theobroma cacao L. (Cocoa) is a preferentially alogamous tropical woody species in the Malvaceae family [1]. The tree produces pods that contain about 40 cocoa beans surrounded by a sweet tasting pulp. When fermented and processed, the beans produce one of the most desired flavours in the world - chocolate. Cocoa was first cultivated in the western region of Nigeria in 1890. Its cultivation gained prominence rapidly in Nigeria such that by 1965, Nigeria became the second largest producer in the world [2]. Nigeria is now the world top seventh producer [3]. The production of cocoa in Nigeria has witnessed a downward trend since the early 1970s due to numerous factors such as ageing trees, ageing farmers, wrong application of recommended agronomic techniques, effects of pests and diseases and deficiencies in macro and micro nutrients in the soils [4]. Soil quality has been known to have a strong effect on cocoa tree growth [5, 6, 7, 8]. Reports of the soil fertility evaluation across cocoa ecologies in Nigeria have shown that phosphorous and potassium is limiting [9] hence, the use of fertilizer has become inevitable. More than 85% of cocoa farmers in Nigeria do not use fertilizers on cocoa [10].
Soil microorganisms participate in the processes that are crucial for long-term sustainability of agricultural systems [11].The rhizosphere, or the soil under the influence of plant roots [12], is

considered one of the most diverse microbial habitats with respect to species richness and community size [13]. The organisms thriving in the rhizosphere encompass a range of different taxa, including prokaryotic and eukaryotic microorganisms and most abundant among these groups are the bacteria. These microorganisms positively affect plant health through a variety of mechanisms, including mineralization of nutrients, suppression of disease, improving plant stress tolerance, and production of phytohormones [14, 15, 16]. Many studies suggest that the Proteobacteria and the Actinobacteria form the most common of the dominant populations (>1%, usually much more) found in the rhizosphere of many different plant species [17]. Plant-microbe interactions in the rhizosphere influence plant health, productivity and soil fertility [18] and the assembly of microbial communities in the rhizosphere can be affected by human activities such as the input of fertilizers and pesticides [19]. As with most crops, nitrogen (N) is the nutrient required in the largest quantities by cocoa and according to Snoeck *et al*. [20], P fertilisation is likely to increase cocoa growth and yield. The use of organic fertilisers and the inclusion of N_2-fixing trees can greatly contribute to nutrient availability in cocoa production. This may be important especially for farmers for whom it is difficult to access inorganic fertilisers, due to problems with supply and/or cost [21, 22]. Organic residues have the advantage over standard NPK fertilisers of adding other nutrients such as Ca, Mg, and micronutrients. They also assist in maintaining soil organic matter.

2. Methodology

2.1 Nursery and seedling preparation

Amelonado variety Tc1-Tc8 pods purchased from Cocoa Research Institute of Nigeria (CRIN) was used. Pods were opened longitudinally with a knife within 3 days of purchase and good beans were selected from the middle only of the pods, the surrounding pulp was removed using saw dust, the beans were washed afterwards. Each bean were singly placed on a moisted tray and covered under humid condition and sprouting was noticed within 24 hr. Then the emerging part of the germinating beans were inserted in the centre of the soil in a pre-filled polythene bag and adequate watering and weeding followed for the 4 month period of cultivation. Seedlings were generated with methods described by Adeyemi *et al.* [23].

2.2 Collection of fertilizers

Poultry droppings were collected from the Farm House, University of Benin, while fresh cow dung was collected from the Cattle Market in Aduwawa, Benin City. The inorganic fertilizer N.P.K 14-14-14 manufactured by Olam Industries was used.

2.3 Manure composting

The compost pile of poultry droppings and cow dung self-heated to temperatures > 55°C in the central core of the pile on a slab for 4 weeks; at 9 weeks the pile was turned for even distribution of heat and sparely watered. The pile reheated to > 50 - 55°C for one week, and then gradually cooled to ambient temperature by 13 weeks. The pile was allowed to cure for an additional 3 weeks before the compost was air-dried and stored in covered containers. Composite samples were obtained according to standard methods [24].

2.4 Application of fertilizer

The fertilizer application rate for cocoa seedling of 10 kg/ha for inorganic fertilizer and 2.5 t/ha for organic fertilizer [25, 26] was applied around the seedling at 1 month after planting (MAP) as described by Ooi and Chew [27].

2.5 Soil sample collection

A 50 g of the sowing soil was collected and the Root Adhering Soil (RAS) of seedlings were collected every month through 4 months after planting (MAP) [28].

2.6 Bacteriological analysis

Bacteriological analysis were carried out on 1g moist soil sample, dispensed into 9ml sterile distilled water in 3 subsequent dilution to give a 1/10 fold dilution. 1ml of the fourth (4^{th}) dilution was dispensed into Nutrient agar by the pour plate methodology. The diluents were triplicated for confirmation and to check distribution of the cells in the diluents. The plates were then incubated at 37^0C for 24hours. After incubation, colonies were counted and the unit expressed in cfu/g.

2.7 Identification of Isolates

Isolates were examined for size, shape, margin, consistency, elevation. Fresh nutrient agar plates were streaked inoculated for pure culture from plates of different colonies of Isolates. Isolates were identified and characterized using cultural, morphological and biochemical tests.

2.8 Molecular identification of bacteria

2.8.1 DNA Extraction

100mg (wet weight) bacterial cells that have been resuspended in 200 ul of water were added to a ZR Bashing lysis tube. Lysis solution of 750 ul was added to the tube, secured in bead fitted with 2 ml tube holder assembly and processed at maximum speed for 5 mins. The ZR BashingBead ™ lysis tube was then centrifuged at > 10,000 x g for 1 min. Up to 400 ul supernantant was transferred to a Zymo-Spin™ IV spin filter in a collection tube and centrifuged at 7,000 x g for 1 min and 1200 ul of bacterial DNA binding buffer was added to the filtrate in the collection tube. Thereafter, 800 ul of the resulting mixture was transferred to a Zymo-Spin™ IIC column in a collection tube and centrifuged at 10, 0000 x g for 1 min and the step repeated again. Next, 200 ul of DNA pre-wash buffer was added to the Zymo-Spin™ IIC column in new collection tube and centrifuged at 10,000 x g for 1 min. Then 500 ul bacterial DNA wash buffer was added to the Zymo-Spin™ IIC column and centrifuged at 10, 0000 x g for 1 min and the Zymo-Spin™ IIC column was transferred to a clean 1.5 ml micro centrifuge and 100 ul of DNA Elution Buffer was directly added to the column matrix. It was centrifuged at 10,000 x g for 30 secs to elute the DNA.

2.8.2 Polymerase Chain Reaction

The DNA was subjected to PCR buffer, $Mgcl_2$, DMSO, DNTPs, Taq and H_2O.
The Primers 16SF: GTGCCAGCAGCCGCGCTAA
16SR: AGACCCGGGAACGTATTCAC were used to amplify the 16S rRNA gene .
Initial denaturation was at 94^0 c for 5 mins and denaturation at same temperature for 30 sec. Annealing was at 54^0 c for 30 sec, extension was at 72^0 c for 45 sec and for 36 circles. Final extension was at 72^0 c for 7 min and hold temperature of 10^0 c. The amplicons from the reaction was loaded on 1.5% agarose gel and the gel picture is attached as PCR. The ladder used was hyper ladder 1 from Bioloine. The expected base pair of the amplicons was around 650bp.Gene AMP PCR system 9700 was used for PCR amplification.

2.8.3 Sequencing

Genetic analyzer was ABI 3500 which was used for sequencing. Sequences of the isolated strains were compared with sequences in GenBank using the alignment search tool (BLAST) [29, 30].

2.9 Statistical analysis

The data collected were analyzed using analysis of variance (ANOVA) and means were separated using Genstat statistical package 10th edition (Turkey test) LSD at the 5% level of significance.

3. Results and Discussion

Table 1: Count of bacterial population

S/No.	Treatment	Time	Bacterial population (cfu/g) x 10^5
1	Cow dung	cd	2.70
2		cd1	4.95
3		cd2	3.45
4		cd3	2.85
5	Control	c	5.25
6		c1	5.05
7		c2	4.25
8		c3	3.95
10	NPK	npk1	3.05
11		npk2	2.35
12		npk3	2.45
13	Poultry manure	pm	7.55
14		pm1	11.7
15		pm2	9.65
16		pm3	8.20

Key:
cd = cow dung manure cd 1,cd 2 and cd 3 = rhizosphere of soil amended with cow dung after 1 months, 2 months and 3 months respectively. Control = sowing soil c1, c 2, c 3 = un-amended soil after 1month , 2months and 3months respectively. Npk 1, 2 and 3 = rhizosphere of soil amended with NPK after 1 month, 2 and 3 months respectively. Pm = compost poultry manure pm1, 2 and 3 = rhizosphere of soil amended with poultry manure after 1 month , 2 months and 3 months respectively.

Table 2: Bacterial isolates

S/No.	Treatment	Time	Isolates
1	Cow dung	cd	*Acinetobacter calcoacetius, Comamonas testosteroni, Burkholderia vietnamiensis*
2		cd1	*Acinetobacter calcoace, Comamonas testosteroni, Lysinibacillus macroides, Burkholderia vietnamiensis, Janthinobacterium lividum, Brevundimonas diminuta*
3		cd2	*Ralstonia pickettii, Acinetobacter calcoacetius, Pseudomonas aeruginosa*
4		cd3	*Acinetobacter calcoacetius, Comamonas testosteroni, Burkholderia vietnamiensis*
5	Control	c	*Ralstonia pickettii, Comamonas testosteroni, Lysinibacillus macroides, Bacillus subtilis*
6		c1	*Bacillus subtilis, Comamonas testosteroni, Pseudomonas aeruginosa, Lysinibacillus macroides, Acinetobacter calcoacetius*
7		c2	*Ralstonia pickettii, Pseudomonas aeruginosa, Comamonas testosteroni, Acinetobacter calcoacetius, Bacillus subtilis*
8		c3	*Pseudomonas aeruginosa, Bacillus subtilis, Comamonas testosterone*

10	NPK	npk1	*Ralstonia pickettii, Comamonas testosteroni, Bacillus subtilis*
11		npk2	*Ralstonia pickettii, Bacillus subtilis, Comamonas testosteroni, Pseudomonas aeruginosa*
12		npk3	*Burkholderia vietnamiensis, Comamonas testosteroni, Bacillus subtilis, Pseudomonas aeruginosa*
13	Poultry manure	pm	*Burkholderia vietnamiensis, , Pseudomonas aeruginosa, Comamonas testosteroni, Bacillus subtilis*
14		pm1	*Pseudomonas aeruginosa, Comamonas testosteroni, Bacillus subtilis*
15		pm2	*Pseudomonas aeruginosa, Comamonas testosteroni, Bacillus subtilis*
16		pm3	*Burkholderia vietnamiensis, Pseudomonas aeruginosa, Comamonas testosteroni, Lysinibacillus macroides*

Table 3: means for bacterial population and isolates

Treatment	Bacteria population	Isolates
Cow dung	44.12a	19.33a
Control	49.50a	**25.75b**
Poultry manure	**60.25b**	**29.75b**
NPK	46.38a	20.75a

Means in same column followed by same letter(s) are not significantly different $P \leq 0.05$ using Turkey Test.

Modern farming practices, such as fertilizer applications can alter soil microbial communities through their impact on various edaphic factors, including soil moisture, pH [31, 32, 33], nutrient availability, organic matter content, and temperature [34, 35, 36, 37].

The bacterial count of all fertilizers before application show compost poultry manure with the highest count (7.55×10^5 cfu/g), followed by compost cow dung (2.70×10^5 cfu/g) while NPK showed no growth. While the soil samples show the highest count in the rhizosphere of cocoa seedlings amended with poultry manure ($8.20 – 11.70 \times 10^5$ cfu/g), followed by the control ($3.95 – 5.05 \times 10^5$ cfu/g) and the NPK amended soil had the lowest count ($2.35 – 3.05 \times 10^5$ cfu/g). The rich composition of poultry manure is probably the reason why it recorded the highest count, while the diet and intestinal digestion that the cow dung underwent might account for the lower bacterial count. The chemical composition of the NPK would be the reason for the zero count recorded in comparison to the organic fertilizers. In comparison to mineral fertilizers, organic fertilizers (e.g., animal manures and compost) have been reported to enhance the bacterial richness (number of species) and lower evenness (relative abundance of taxa) of soil communities [38, 39]. In a study, O'Brien [40] reported that organic fertilizer treatment was found to have a significant effect on the overall bacterial abundances in the rhizosphere soils.

The isolated organisms as shown in Table 2 were members of the phyla Proteobacteria and Firmicutes, and they include *Burkholderia* sp., *Pseudomonas* sp., *Comamonas* sp., *Lysinibacillus* sp., *Bacillus* sp., *Acinetobacter* sp., *Janthinobacterium* sp., *Ralstonia* sp., *Brevundimonas* sp.. Proteobacteria is the predominant phylum in rhizosphere, this may be due to their rapid growth rates and, because the nutrient-rich environment is suitable for this phylum or certain classes within this phylum [41]. They are mostly Gram-negative and many are responsible for nitrogen fixation and polycyclic aromatic hydrocarbons. These findings were consistent with previous studies on bacterial communities in soil [42, 43], where the major soil phyla comprised of Proteobacteria, Actinobacteria, Acidobacteria, Bacteroidetes, Firmicutes and Plantcomycetes. The heterotrophic Bacteroidetes, and Firmicutes are related to the decomposition of soil organic matter [44]. Similarly, Li *et al*., 2014, Yang *et al*., 2017 and Goldfarb *et al*., 2011 [45, 46, 47] in their study reported proteobacteria as the most abundant phylum in soil. Also, *Bolhuis et al.* [48] found

that significant growth-related dynamic changes in bacterial community structure were mainly associated with phylum Bacteroidetes, Proteobacteria and Actinobacteria (mainly genera *Burkholderia*, *Flavisolibacter* and *Pseudomonas*). Members of *Burkholderia* were enriched in the rhizosphere, possibly due to their versatile abilities to utilize root metabolites, degrade aromatic compounds [49] and produce anti-microbial substances. The Use of manure can increase the long term sustainability of agriculture and its impact on global climate change. Also, high transport cost, constant price increase and scarcity of has not enabled many farmers to use inorganic fertilizer. While organic manure is cheaper, the knowledge of this alternative either singly or in combination with inorganic fertilizer is still not wide spread. A study by Adejobi *et al.* [50] showed that organic fertilizer materials positively and significantly affected the growth parameters of cocoa seedlings such as plant height, stem diameter, number of leaves per plant and leaf area relative to control. Similarly, poultry manure and organo-mineral fertilizer has been reported to perform better on growth of cocoa seedlings than inorganic NPK [51]. However, a finding also indicated that there was no significant difference between annual yam yields per hectare, using organic and inorganic fertilizers [52].

4. Conclusion

The benefits of application of poultry manure to agricultural soil are obvious as the results show over cow dung and much more over inorganic fertilizer.

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